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Bioscience Research

Print ISSN: 1811-9506 Online ISSN: 2218-3973

Journal by Innovative Scientific Information & Services Network

RESEARCH ARTICLE BIOSCIENCE RESEARCH, 2019 16(3): 2979-2992. **OPEN ACCESS**

Biochemical properties and thermostability of bacterial phytase

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Phytase (E.C. 3.1.3.8) is one of the hydrolytic enzymes. The enzyme has many industrial applications such as dephytinization of wheat flours. was purified from *E. coli* and *S. aureus*. The best substrates were sodium phytate and 1-naphthyl phosphate. The enzyme was induced by thioglycolate, dithiothreitol (DTT), gibberellic acid and benzyl amino purine (BAP). Phytase from both sources was inhibited by fluoride, cyanide and malonate. The enzyme was also inhibited by three chelating agents o-phenanthroline, α-α-dipyridyl and EDTA. The enzyme was activated by Ca^{+2} but the other metal ions were inhibitors. The amino acids cysteine, methionine and glycine were activators, but the other amino acids inhibited the enzyme. Phytase from *E. coli* was more resistant to the digestive enzymes pepsin and trypsin than that from *S. aureus.* Phytase was labile at 60°C; however trehalose and sorbitol provided the enzyme with appreciable thermostability.

Keywords: Phytase, *E. coli, S. aureus*, Digestive enzymes, Thermostability, Phytohormones, Amino acids.

INTRODUCTION

Phytase is chemically known as myo-inositol hexa kisphosphate phosphohydrolase (E.C.3.1.3.8) and it belongs to a special class of phosphor mono esterases. Phytase catalyzes the stepwise phosphate splitting of phytic acid or phytate to lower inositol phosphate esters and inorganic phosphate (Kumar et al., 2010).

Phytases have been studied most intensively in the seeds of plants (Greiner, 2002), and they are found also in microorganisms and in some animal tissues (Konietzny and Greiner, 2002). The first commercial phytase products were launched into market in 1991 (Haefner et al., 2005). Natural phytases are present in plants to carry out the dephosphorylation of phytate complexes (Jorquera et al., 2008).

Therefore, phytases are considered to be potential candidate for enhancing the nutritional quality of phytate-rich foods and feed (Kumar et al., 2013). Phytases are not only used as animal feed additive in diets for monogastric animals but there is great potential for the use of this class of enzymes in processing and manufacturing of food for human consumption (Kumar et al., 2010).

The bacterial phytases possess several characteristics that make them important in enzyme industry. These include broad spectrum of temperature tolerance, wide range of pH high catalytic efficiency, and greater resistance to pepsin. A good example is the phytase-associated class of *E. coli*, which enhances the overall availability of phosphate from phytate (Passos et al., 2015). The strain BL21 (DE3) of *E. coli* can produce phytase up to 20 % of total soluble protein under T7 promoter (Kim et al., 1998).

Many phytase producing bacteria have also been isolated, screened and reported for their ability to produce phytases e.g. *Pseudomonas sp*. (Richardson and Hadobas, 1997), *Bacillus sp*. (Choi et al., 2001), *E. coli* (Greiner et al., 1993)

and anaerobic rumen bacteria, particularly in *Mitsuokella jalaludinii* (Lan et al., 2002).

Microbial phytases are easily produced and extracted when synthesized extracellularly in a culture medium, and generally, they may be synthesized by the same microbial starter used for food processing. Genetically modified strains of bacteria such as *Bacillus subtilis, E. coli, Klebsiella sp*., and *Bacillus Amyloliquefaciens*; yeasts such as *Schwanniomyces castellii, Hansenula polymorph, Schwanniomyces occidentalis, and Rhodotorula gracilis*. Also, fungi such as *Aspergillus ficuum* and *Aspergillus niger* are some of the most important species used for the production of microbial phytases (Pandey et al., 2001).

The present work aimed to study the characteristics and thermostability of the purified phytase from *E. coli* and *S. aureus*.

MATERIALS AND METHODS

Experimental organisms

The experimental strains which used in this study were *E. coli* (ATCC 8739) and *S. aureus* (ATCC 6538). They were obtained from the American Type Culture Collection (ATCC, Minnesota, USA).

Growth medium

The following media were used for isolation and identification of the strains.

1-Tryptic soy broth (TSB) was obtained from Sigma- Aldrich. It contained tryptone (Pancreatic Digest of Casein) 17.0 g, soytone (Peptic Digest of Soybean) 3.0 g, glucose (= dextrose) 2.5 g, sodium chloride 5.0 g, and dipotassium phosphate 2.5 was dissolved in 1000 ml distilled water. The final pH was 7.3 at 25 °C.

2-Reasoner's 2A agar (R2A) (Van der Linde et al., 1999). It contained 0.5 g proteose peptone , 0.5 g casamino acids, 0.5 g yeast extract, 0.5 g soluble starch, 0.5 g dextrose, 0.3 g dipotassium phosphate, 0.05 g magnesium sulphate per $7H_2O$ and 15.0 g agar dissolved in 1000 ml distilled water. The final pH was 7 at 25 °C.

Assay of phytase enzyme

Phytase was assayed according to the methods of Scott and Loewus (1986) by measuring the released inorganic phosphorus from sodium phytate at 25°C for 30 min. The reaction mixture contained 3.0 ml 100 mM Tris-HCl (pH 7.0), 100 µl of enzyme extract, 0.2 ml 15 mM $CaCl_2$ and 0.5 ml 2.0 mM sodium phytate. The enzyme reaction was terminated by the addition of 0.2 ml 10 % (w/v) TCA. The mixture was then centrifuged at 5000 rpm for 20 min to remove the precipitated protein. The resulting supernatant was analyzed for inorganic phosphate. One unit (U) of phytase is defined as the amount of enzyme required for production of one µmol of inorganic phosphorus at 25°C per min.

Effect of incubation time on purified phytase activity

Both free and immobilized enzymes either on chitosan or alginate were incubated for different periods of time (20, 40, 60, 80, 100 and 120 min) followed by measuring the enzyme activity.

Effect of different substrates on the activity of purified phytase

The activity of purified phytase from both *E. coli* and *S. aureus* was assayed using various substrates including sodium phytate, 1-naphthyl phosphate, phenyl phosphate, glycerol-2-phosphate, *and p*-nitrophenyl phosphate. The activity was calculated relative to the activity with sodium phytate where the activity is considered 100 %.

Effect of thiol compounds on activity of purified phytase

The effect of thiol compounds such as dithiothreitol (DTT) and thioglycolate were investigated at 100, 200, 300, 400, and 500 µmol in the reaction mixture, followed by the enzyme assay as mentioned before.

Thermostability of phytase

Thermostability of the purified phytase was assessed by pre-incubation enzyme without sodium phytate as substrate at 60ºC. The residual enzyme activity was determined after 20, 40, 60, 80, 100 and 120 min. The residual activity of phytase was expressed as a percentage relative to the initial enzyme activity. The effect of 10 mM trehalose, glycerol, sorbitol and mannitol on the thermostability of the purified phytase was tested at 60°C for 20, 40, 60, 80, 100 and 120 min followed by measuring the percentage of remaining activity.

Effect of various anions on purified phytase activity

The effect of Na-fluoride, Na-cyanide and Na-malonate at various concentrations (0.2, 0.4, 0.6, 0.8, and 1.0 mM) on purified phytase was tested

and the relative activity and inhibition percentage were calculated.

Effect of metal ions on the activity of purified phytase

Seven metal ions were tested in the present investigation regarding to their effect on phytase activity. They were Mg^{2+}, Mn^{2+}, Cu^{2+}, Zn^{2+}, Ca^{2+}, Hg^{2+}, Cd^{2+}, and Ni^{2+}. These metal ions were tested in the reaction mixture as chloride salts at 5 and 10 mM. The phytase activity was measured as mentioned previously.

Effect of digestive enzymes on purified phytase activity

The effect of trypsin and pepsin using 10 units from each of them on purified phytase activity was studied for 5 days. The relative activity and IC_{50} values were determined as mentioned before.

Effect of amino acids on purified phytase activity

The influence of cysteine as amino acid containing thiol group on purified phytase was tested at various concentrations (30, 60, 90, 120, and 150 µmol). Also, glycine and methionine were tested at 100, 200, 300, 400 and 500 µmol in the reaction medium followed by measuring the enzyme activity.

Effect of growth regulators on purified phytase activity

The effect of benzylamino purine (BAP) on phytase activity was investigated at 50, 75, 100, 125, and 150 µmol and gibberellic acid (GA_3) was investigated at various concentrations (20, 40, 60, 80, and 100 µmol) in the reaction mixture followed by measuring the activity of phytase.

Effect of chelating agents on purified phytase activity

The three chelating agents namely α-α-dipyridyl, *O*- phenanthroline and EDTA were investigated regarding their effect on purified phytase activity. Each of the three compounds was tested at various concentrations (5, 10, 15, 20, 25, and 30 mM) followed by measuring enzyme activity.

RESULTS

Effect of incubation time on phytase activity from *E. coli and S. aureus*

The effect of incubation time on phytase activity was tested throughout 120 min. The enzyme activity was measured every 20 min. The results in Fig. 1 reveal that the optimal incubation time was 80 min and 60 min for *E. coli* and *S. aureus*, respectively.

Effect of different substrates on phytase activity from *E. coli and S. aureus*

The effect of various substrates on phytase activity was investigated. They were sodium phytate, phenyl phosphate, 1-naphthyl phosphate, *p*-nitrophenyl phosphate, and glycerol-2-phosphate. The results in Fig. 2 show that the phenyl phosphate was the best followed by 1-naphthyl phosphate, *p*-nitrophenyl phosphate, and glycerol-2-phosphate.

Thioglycolate and dithiothreitol (DTT) are thiol compounds. The activity of phytase from both *E. coli* and *S. aureus* was determined under the influence of thioglycolate at various concentrations (100, 200, 300, 400, and 500 µmol). The results in Fig. 3a indicate activation of the enzyme from both organisms by thioglycolate. The activation percentage for *E. coli* enzyme ranged between 12.7 % and 60 %. However, the activation percentage for *S. aureus* was confined between 17.3 % and 52 %.

The results in Fig. 3b show that DTT activated phytase with 50 % from *E. coli* at 300 µmol then declined at the higher concentration. However, in case of *S. aureus* the activity of phytase increased up to 400 µmol where the activation percentage was 40.8 %. However, phytase from *S. aureus* was activated with 54.5 % at 300 µmol then declined at 400 µmol and 500 µmol, respectively.

Effect of trehalose and sorbitol on thermostability of phytase from *E. coli and S. aureus* at 60ºC

The results in Fig. 4a indicate that trehalose protected the enzyme against heat inactivation at 60ºC form *E. coli* and *S. aureus*. The $T_{0.5}$ was calculated and its values were 236.3 min and 115.8 min for *E. coli* and *S. aureus*, respectively. Glycerol supported the enzyme activity against heat inactivation (Fig. 4b). The $T_{0.5}$ values were 134.4 min and 55.7 min for phytase from *E. coli* and *S. aureus*, respectively. Sorbitol protected partially phytase from both organisms (Fig. 4c). The $T_{0.5}$ was calculated and its values were 281.4 min and 148 min for the enzyme from the two organisms, respectively. Also, mannitol expressed partial protection against heat inactivation for the enzyme from both organisms at 60ºC. The $T_{0.5}$ values were 364.7 min and 148 min for the two organisms, respectively.

Effect of metal ions on activity of purified phytase from *E. coli and S. aureus*

The experiment was carried out in the presence of $MgCl_2$, $MnCl_2$, $CuCl_2$, $CuCl_2$, $CaCl_2$, $HgCl_2$, $CdCl_2$, and $NiCl_2$. Each of these metal chlorides was tested at 5 and 10 mM. The results in Fig. 5 show that $CaCl_2$ induced the highest activity expressed an activity of 19.4 Umg^{-1} protein compared with 14.5 Umg^{-1} protein for the control sample. The other cations expressed various inhibitory effects.

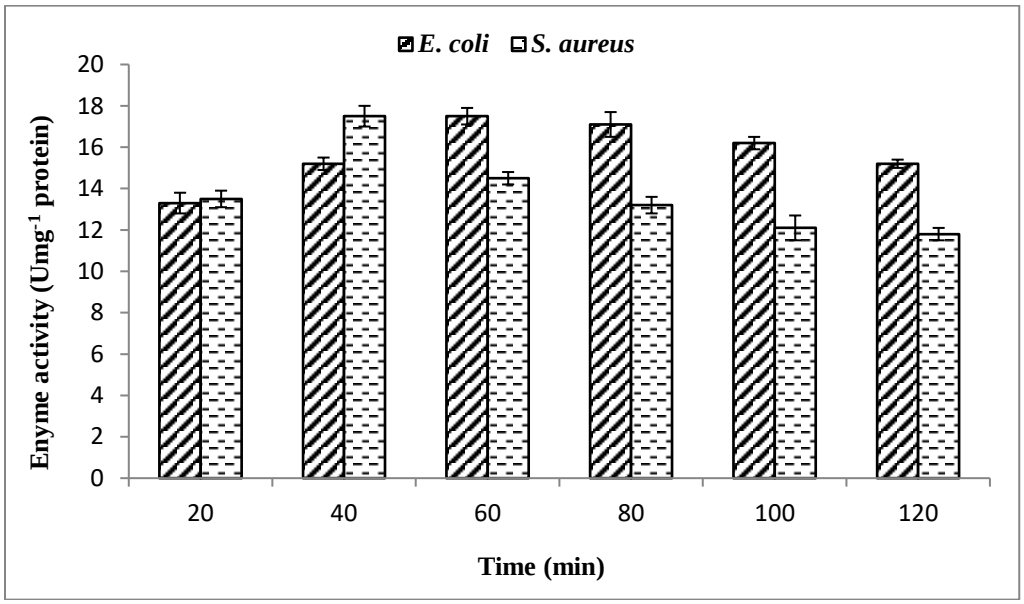


Figure. 1: Effect of incubation time on phytase activity from *E. coli* and *S. aureus*.

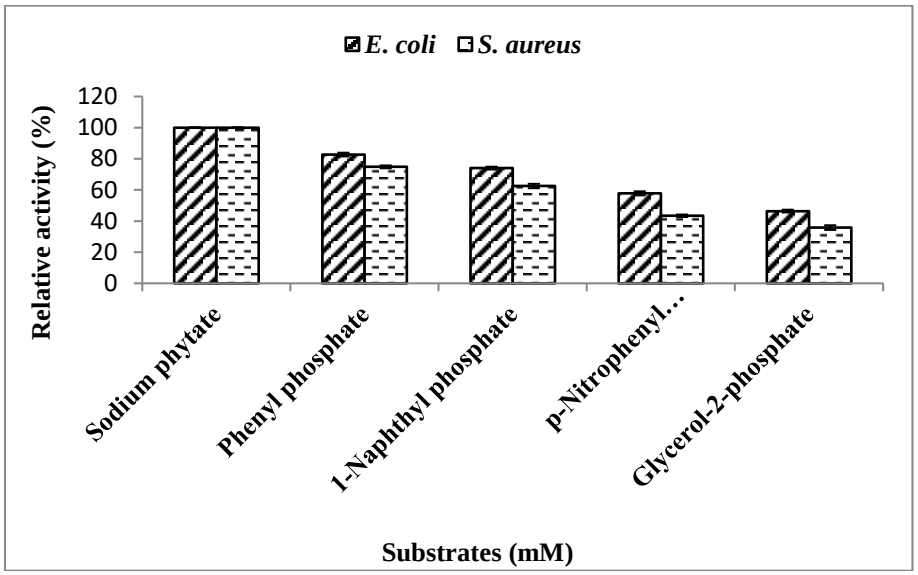


Figure. 2: Effect of different substrates on phytase activity from *E. coli* and *S. aureus*.

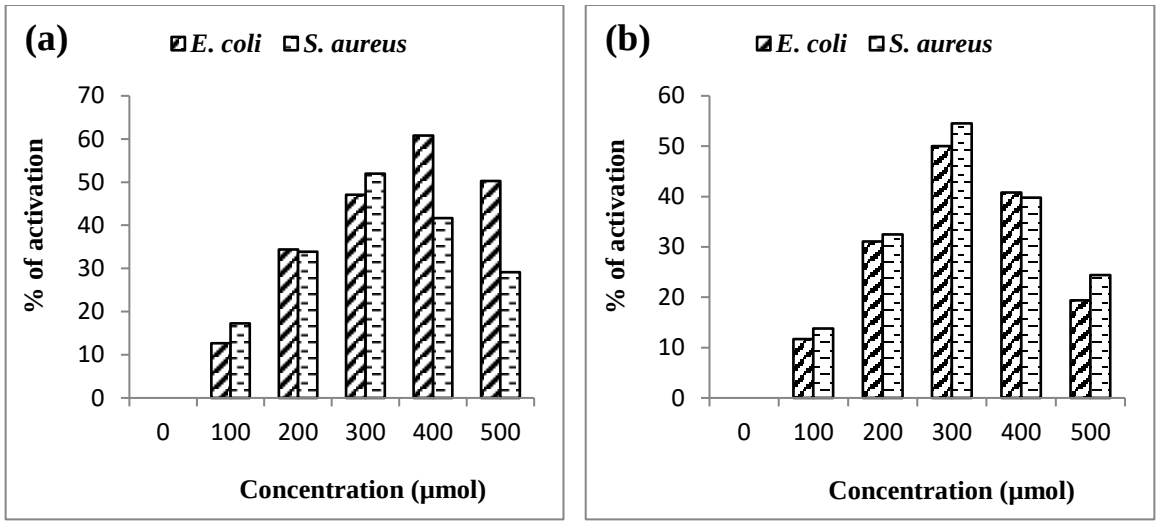
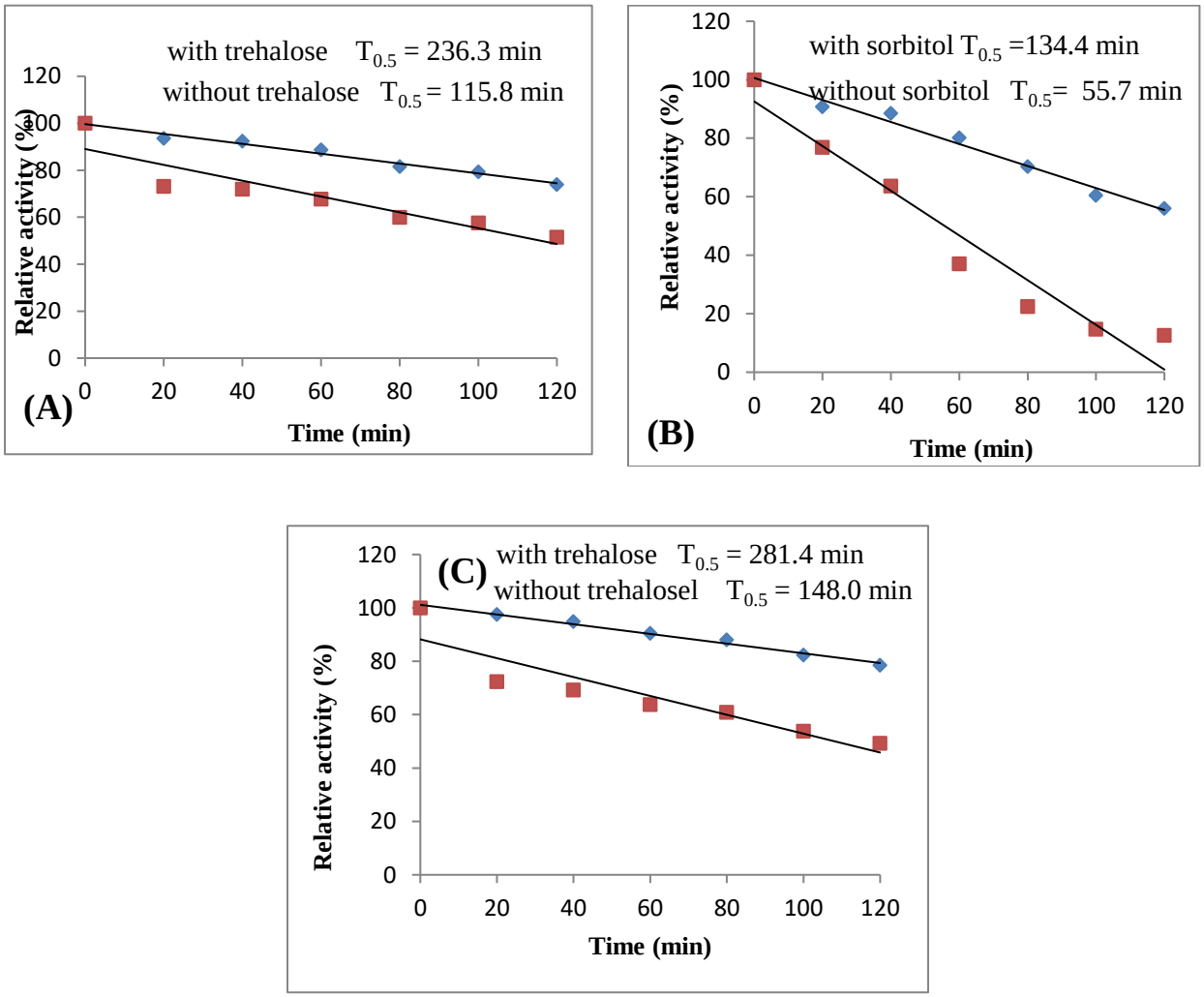


Figure. 3: The activation percentage of purified phytase by thioglycolate and dithiothreitol (DTT).



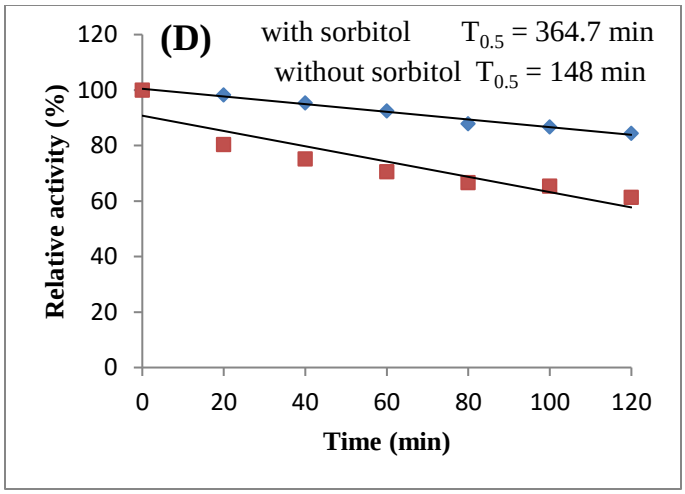


Figure. 4: Thermostability of phytase from *E.coli* (A & b) and *S. aureus* (C & D) at 60°C in presence of trehalose and sorbitol.

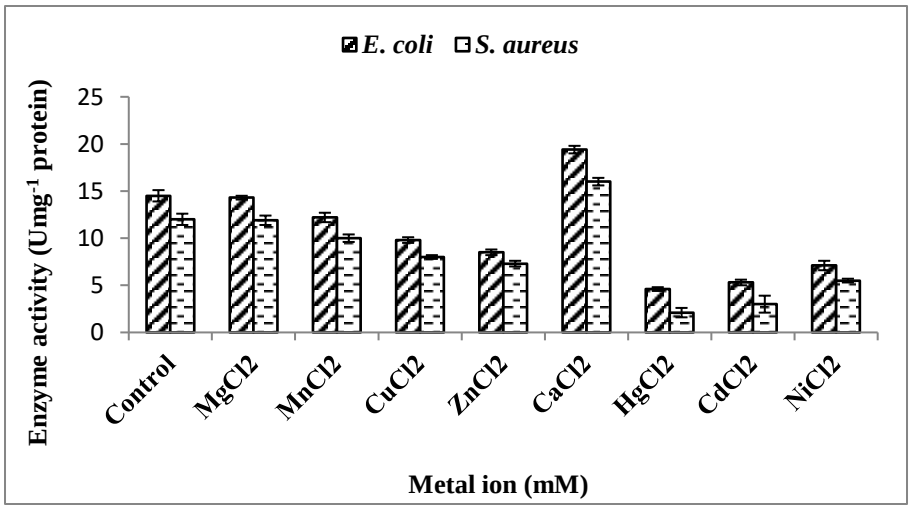
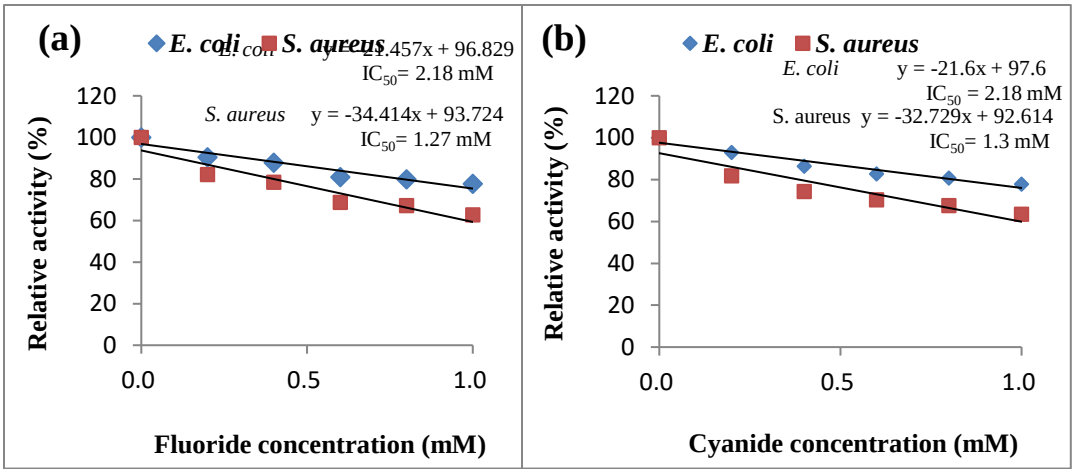


Figure. 5: Effect of metal ions on activity of purified phytase from *E. coli and S. aureus*



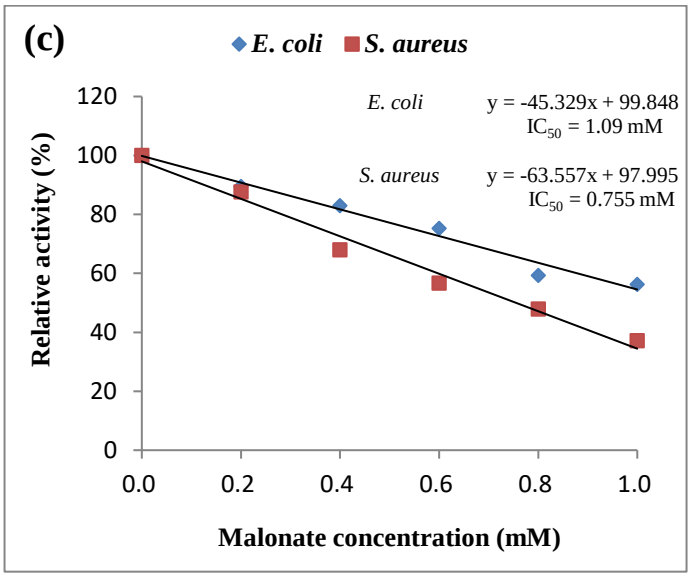


Figure 6: Effect of various anions on phytase activity. (a) fluoride, (b) cyanide and (c) malonate.

Effect of various anions on purified phytase from *E. coli and S. aureus*

The effect of sodium fluoride, sodium cyanide and sodium malonate on phytase activity was investigated. Fig. 6a show that there was a gradual decrease in the enzyme activity depending on the concentration of the sodium fluoride. IC_{50} of sodium fluoride was calculated and its values were 2.18 mM and 1.27 mM for the two organisms, respectively. There is continuous reduction in the enzyme activity under the effect of sodium cyanide (Fig 6b). IC_{50} of sodium cyanide were calculated and its values are 2.18 mM and 1.3 mM for the two organisms, respectively.

Phytase activity was reduced in presence of sodium malonate (Fig 6b) depending on the concentration of malonate. IC_{50} values of sodium malonate were 1.09 mM and 0.755 mM for the enzyme from *E. coli* and *S. aureus*, respectively.

Effect of trypsin and pepsin on purified phytase from *E. coli and S. aureus*

This experiment aimed to investigate the influence of trypsin or pepsin on phytase from *E.coli* and *S. aureus*. The results in Fig. 7a showed continuous reduction in phytase activity with increasing the incubation time with trypsin. It was observed that phytase from *S. aureus* was strongly inhibited than that from *E. coli*. The $T_{0.5}$ values for the enzyme were 7.28 and 3.1 days for the phytase from *E.coli* and *S. aureus*, respectively.

The results in Fig. 7b indicated that the activity decreased gradually with the time after incubation with pepsin. The inhibition percentage was higher in phytase from *S. aureus* than that of *E. coli*. $T_{0.5}$ values were 7.1 and 4.3 days for the enzyme from *E. coli* and S*. aureus*, respectively.

Effect of various amino acids on phytase activity from *E. coli and S. aureus*

The influence of amino acids on phytase from both organisms was investigated using methionine, cystine, cysteine, glycine, arginine and glutamine. The results in Fig. 8 indicated that methionine, cysteine and glycine were activators cysteine increased the activity with 37.7 % and 28.9 % for *E. coli* and *S. aureus*, respectively. However the remaining amino acids were inhibitors for phytase from *E. coli* and *S. aureus*.

Effect gibberellic acid (GA_3) and benzylamino purine (BAP) on purified phytase from *E. coli and S. aureus*.

The results in Fig 9a indicated continuous activation of phytase activity from *E. coli* depending on the concentration of GA_3. The activation of phytase from *E. coli* confined between 8.6 % at 20 µmol and 63 % at 60 µmol then reduced to 46.5 % and 33.7 % at 80 µmol

and 100 µmol for phytase from *E. coli*. The results show that the percentage of activation ranged between 33.3 at 20 µmol and 98.4 % at 60 µmol then declined to 93.6 and 60.3 % at 80 µmol and 100 µmol for *S. aureus* enzyme.

The results in Fig 9b indicated that BAP increased phytase activity depending on the concentration up to 100 µmol where the activity was activated by 46.6 % and 24.1 % for *E. coli* and *S. aureus* respectively. At the higher the activity declined gradually reaching 39.7 % and 11.7 % for *E. coli* and *S. aureus* at 125µmol.

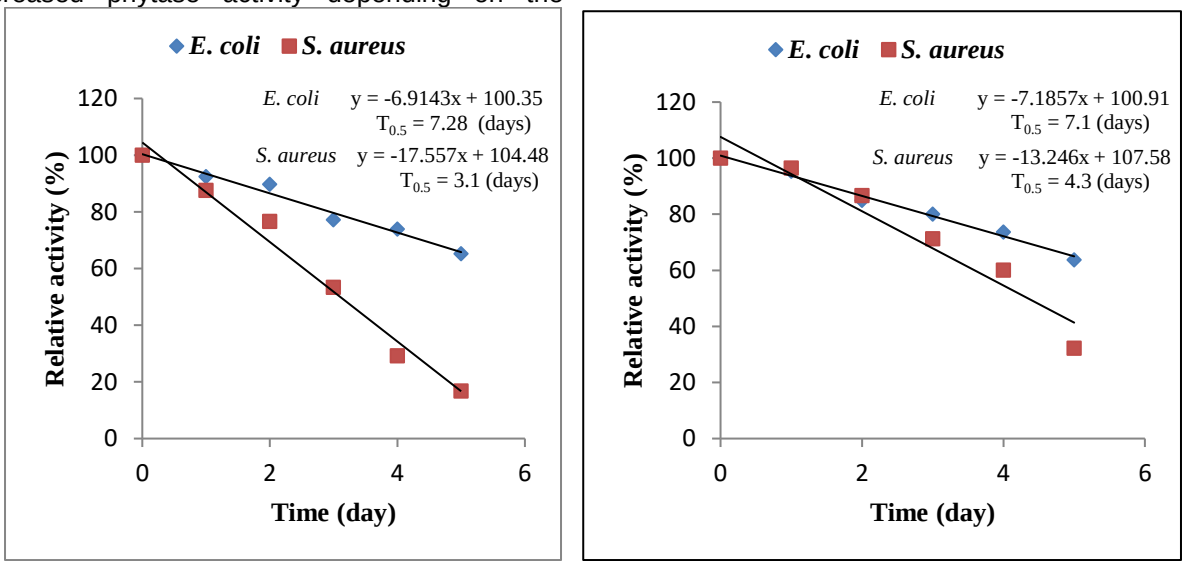


Figure. 7: Effect of 10 U of trypsin (a) and pepsin (b) on phytase activity from *E. coli* and *S. aureus*.

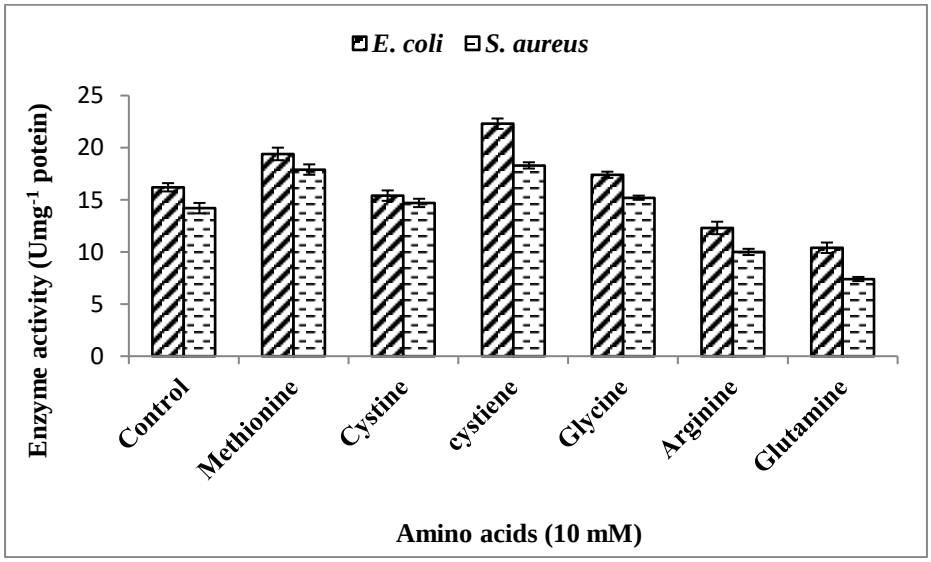


Figure. 8: Effect of various amino acids on phytase activity from *E. coli* and *S. aureus*.

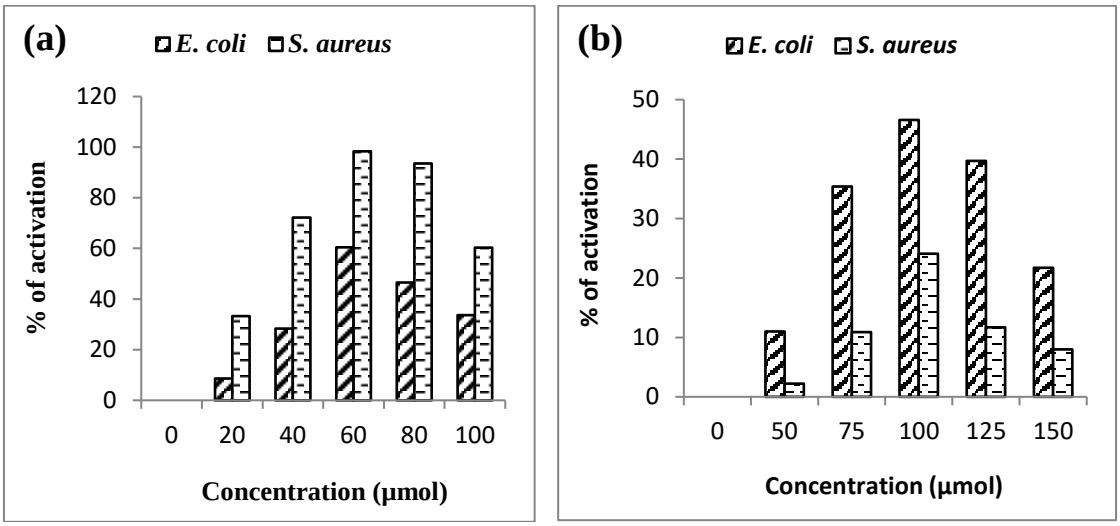
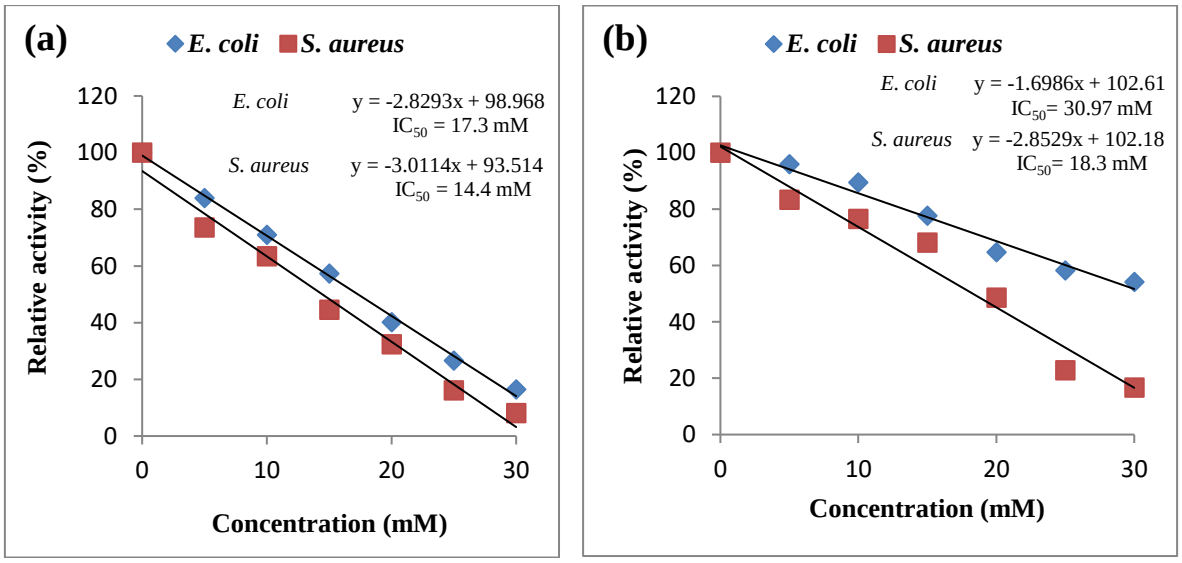


Figure. 9: The activation percentage of phytase activity under treatment with, (a) gibberellic acid (GA_3) and (b) benzylamino purine (BAP).



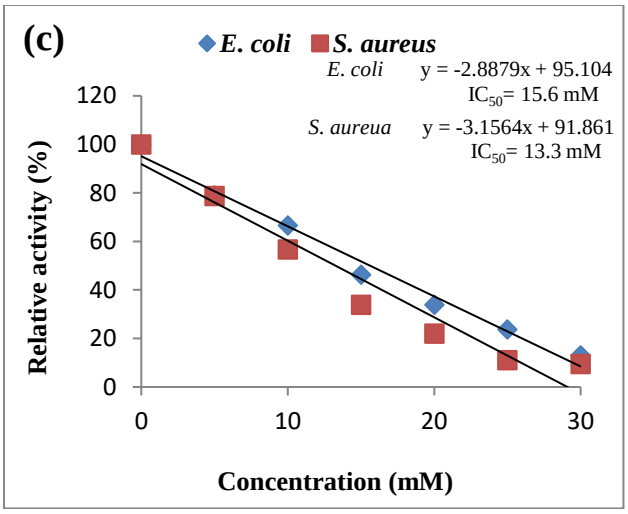


Figure. 10: Effect of chelating agents: (a) α-α- dipyridyl, (b) *O*- phenanthroline and (c) ethylenediaminetetraacetate (EDTA) on phytase activity from *E. coli* and *S. aureus*.

Effect of α-α- dipyridyl, O- phenanthroline and ethylenediaminetetraacetate (EDTA) on purified phytase from *E. coli and S. aureus.*

Fig 10a showed that α-α- dipyridyl was inhibitor for phytase from *E. coli* and IC_{50} were 17.3 and 14.4 mM. *O*- phenanthroline (Fig. 10b) inhibited phytase and IC_{50} were 31 mM and 18.3 mM for the two organisms in the same order. EDTA (Fig.10c) inhibited phytase from both organisms and IC_{50} values were 15.6 mM and 13.3 mM, respectively.

Discussion

The optimal time of incubation was 60 min. The result illustrates the relationship between the time of incubation and the enzyme activity, has two distinct regions. Firstly, in initial period of time, the amount of substrate, which has been transformed, seems to be directly proportional to the length of time for which the reaction has been proceedings.

After the initial period, the rate of reaction starts to decrease and that indicate the substrate is present in excess, the explanation of this phenomenon is the progressive less of enzyme activity after a period of time. This may be due to the effect of heat of incubation on the tertiary structure of the enzyme or to the formation of some product or side-product of the reaction, which inhibits the enzyme (Lizotte et al., 1990).

The present results showed that phytase has broad substrate specificity to some extent with higher activities with sodium phytate. Phenyl phosphate, 1-naphthyl phosphate, *p*-nitrophenyl phosphate, glycerol-2-phosphate expressed little activities compared with sodium phytate. These results are in agreement with the findings of Scott and Loewus (1986).

DTT activated phytase in addition DTT was reported to protect the loss of enzyme activity which occurs by the oxidation of sulfhydryl groups (Alliegro, 2000; El-Shora and Abo-Kassem 2000; El-Shora and Metwally, 2009). This may indicate the SH group of phytase was protected during the incubation time. Also, it is possible that these thiol compounds may cause lowering of the Km of the enzyme to its substrate and thereby activating the enzyme.

Including thioglycolate in the reaction medium of phytase resulted in enhancing phytase activity. This may be due to protection of –SH group by thioglycolate which enhanced the activities of other enzymes including protease (El-Shora et al., 2016a; El-Shora et al., 2017).

Phytase from both E. coli and S. aureus was denaturated at higher temperature over the optimal one. The heat stability of the enzyme is affected by two factors. The first one is the primary structure of the enzyme molecule that provides a compact structure, that is not denatured by a change in the external environment. Also, disulfide bridges and other bonds provide a high resistance both to heat inactivation and chemical denaturation. Secondary, specific components such as divalent cations, can stabilize the molecule (Ozturk, 2001).

Trehalose as disaccharide protected phytase activity against thermal denaturation. So, trehalose is reported as an effective protector for many other enzymes against thermal inactivation (El-Shora et al., 2014; El-Shora et al., 2016b; El-Shora et al., 2018). Trehalose participates in protecting proteins through stabilizing their most compact structure, thus it reduces the backbone movements away from the fully folded state (Sola-penna and Meyer-Fernandes 1998; Nirmal and Laxman, 2014).

The role of sorbitol as polyol on enzyme stabilization is a water-structure marker, which depresses the hydration of the protein. The polyol molecules are preferentially excluded from the surface layer of the protein molecule and the water shell around the protein molecules is preserved, so that the conformation of the protein become more rigid (Longo and Combes, 1999). As general, polyols are capable to maintain solvophobic interaction and able to form hydrogen bonds which play the main role in supporting the conformation of the protein and support the protein stabilization (Lozano et al., 1994; Christainsen and Nielsen, 2002).

It was reported that the ability of any metal to satisfy the binding interactions of the metal-ion binding sites depends on the atomic size, expressed as the effective radius of the hydrated metal ions. It was shown that affinity of the enzyme for divalent cations decreases with decreasing ionic radii since Ca^{2+} (0.099 nm) which has larger radius compared to Mg^{2+} which has small radius (0.072nm) (Hutadilok-Towatan et al., 1999).

A reason for an increase in the activity of enzyme in the presence of calcium (Ca^{2+}) may be due to stabilization of enzyme in its active conformation rather than it being involved in the catalytic reaction. It probably acts as a salt or ion bridge via a cluster of carboxylic groups as has been suggested for subtilisins and thereby maintains the rigid conformation of the enzyme molecule (Strongin et al., 1978).

Since Ca^{2+} binds at noncatalytic sites it may be structural requirement for phytase enzyme. Ca^{2+} was found to serve in modulation of some hydrolytic enzymes (Palmer, 1985).

Ni^{2+}, Zn^{2+}, Cd^{2+} and Hg^{2+} inhibited phytase from both organisms and affect the enzyme activity. These results are in agreement with those of other investigators (Scott and Loewus 1986; Hayes et al., 1999). Also, the inhibition of phytase by Hg^{2+} reveals that sulfhydryl group is essential for phytase catalysis. It is difficult to determine whether the inhibitory effect of various metals is due to direct binding to the enzyme, or whether the metal ions from poorly soluble complexes with phytic acid and therefor decrease the active substrate concentration.

Fluoride, cyanide and malonate inhibited phytase in the present work and fluoride was the strongest inhibitor. This inhibition is in harmony with the findings of Gibson and Ullah (1988).

Also, the present results indicate that phytase activity was reduced in the presence of pepsin and trypsin. However, Yi and Kornegay (1996) reported that yeast phytase lost most of its activity in presence of pepsin which may reveal that phytase from marrow cotyledons is more resistant to pepsin than that of the yeast enzyme.

Cysteine is amino acid increased the activity of phytase increased up to 25.9 Umg^{-1} protein at 500 µmol glycine for E. coli enzyme and 20 Umg^{-1} protein at 400 µmol for S. aureus enzyme. The activation may be due to increasing formation of E-substrate complex. However, higher concentration may hinder the formation of this complex. Cysteine activated other enzymes such as acid phosphatase and asparaginase (El-Shora and Metwally, 2009; Warangkar and Khobragade, 2010). Also cysteine activated protease from *Pterocadia capillacea* (El-Shora et al., 2016c). This may indicate that SH-group of the enzyme was protected during the incubation time. Also, it is possible that these thiol compounds may lower the K_m of the enzyme to its substrate and thus activating the activity.

Both GA_3 and BAP induced phytase from E. coli and S. aureus in the present work. In support, GA_3 induced other enzymes such as phytase (Gabard and Jones, 1986), Also, GA_3 activated other enzymes such as phosphoenolpyruvate carboxylase (Bihzad and El-Shora, 1996), NADH-glutamate synthase (El-Shora, 2001a), phenylalanine ammonia lyase (El-Shora, 2002) and isoperoxidase (Perez and Gomez, 1998; El-Shora, 2002). In addition, GA_3 increased the activities of sucrose synthase and sucrose phosphate synthase (Kaur et al., 2000).

EDTA, O-phenanthroline and α,α-dipyridyl inhibited phytase from E. coli and S. aureus. The inhibition of phytase activity by these compounds revealed that phytase is metalloenzyme. Furthermore, these compounds inhibited other enzymes such as asparaginase (El-Shora et al., 1993) and glucose oxidase (Sabry, 2014). Also, EDTA was also inhibitor for *Chaetimium globosum, Gongronella, Pencillium janthinellum, and Capsicum annuum l*eaves (Shehata and Abd

El Aty, 2015). These results revealed that the chelating agent react with the metal ion leading to the formation of stable complex and suggest that phytase is a metalloenzyme.

CONCLUSION

Cysteine, methionine and glycine were activators for phytase. Phytase from *E. coli* and *S. aureus* was more resistant to the digestive enzymes such as pepsin and trypsin. Trehalose and sorbitol as polyols provided phytase with appreciable thermostability which is important in industrial applications.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

ACKNOWLEGEMENT

The authors thank the Department of Botany in Mansoura University, Egypt for introduced facilities to do the present work

AUTHOR CONTRIBUTIONS

El-Shora designed, performed the experiments, interpreted the data and wrote the manuscript. Also, Salwa and El-Solia carried out some of the experiments. All authors read and approved the final version.

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